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(71) Applicant *(for all designated States except US)*: **IMMUSOL, INC.** [US/US]; 3050 Science Park Road, San Diego, CA 92121 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **TRITZ, Richard** [US/US]; 9515 Genesee Avenue, #127, San Diego, CA 92121 (US). **WELCH, Peter, J.** [US/US]; 4689 E Talmadge Drive, San Diego, CA 92116 (US). **BARBER, Jack, R.** [US/US]; 11987 Cameridge Place, San Diego, CA 92128 (US). **ROBBINS, Joan, M.** [US/US]; 10265 Pinetree Drive, San Diego, CA 92131 (US).

(74) Agents: **MCMASTERS, David, D.** et al.; Seed And Berry LLP, Suite 6300, 701 Fifth Avenue, Seattle, Wa 98104-7092 (US).

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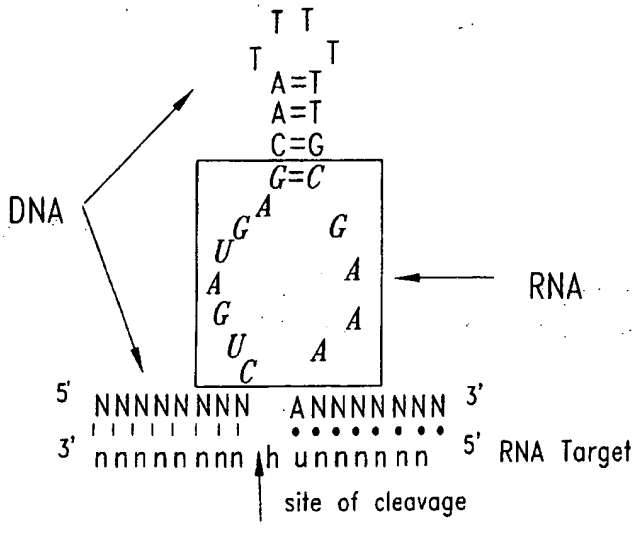

(57) Abstract: As an effective therapy for restenosis, this invention provides ribozymes and ribozyme delivery systems useful to treat or prevent restenosis. Methods of producing ribozymes and gene therapy utilizing these ribozymes also are provided.

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RIBOZYME THERAPY FOR THE TREATMENT AND/OR PREVENTION OF RESTENOSIS

TECHNICAL FIELD

The present invention relates generally to therapeutics, and more specifically, to compositions and methods which may be utilized in the treatment and/or prevention of restenosis.

BACKGROUND OF THE INVENTION

In 1992, an excess of 300,000 angioplasties were performed in the United States. Restenosis is a major complication following angioplasty, occurring in 30%-60% of patients. Indeed, restenosis is the single most significant problem in interventional cardiology and costs the health care system in excess of $ 1 billion per year.

Restenosis following angioplasty is the result of local vascular injury, and is characterized by the local infiltration of platelets and macrophages, and local activation of the clotting system. These factors result in the elaboration of a number of biologic mediators of smooth muscle cell (SMC) migration and proliferation. These SMCs migrate into the vascular intima and begin to proliferate and produce extracellular matrix (ECM), resulting in the formation of a fibrocellular mass which can obstruct blood flow. Further, injury has been shown to induce the expression of a variety of oncogenes that are believed to play a role in the cellular response to this injury.

Thus, a need exists for an effective therapy to prevent and treat restenosis. The present invention satisfies this need and further provides other related advantages as well.

SUMMARY OF THE INVENTION

As an effective therapy for restenosis, this invention provides ribozymes and ribozyme delivery systems which are able to inhibit abnormal smooth muscle cell

proliferation in vascular tissue, and in particular, are suitable for treating or preventing restenosis. Methods of producing ribozymes and gene therapy utilizing these ribozymes also are provided.

Accordingly, in one aspect the present invention ribozymes having the ability to inhibit a cyclin or cell-cycle dependent kinase, with the proviso that said cell-cycle dependent kinase is not CDK1, PCNA or Cyclin B1. Particularly preferred cyclins or cell-cycle dependent kinases include CDK4, CDK2, and Cyclin D. Preferably, the ribozyme is a hammerhead or hairpin ribozyme, representative examples of which recognize the target site sequences set forth below, and in the Examples. Representative recognition sites are provided in Sequence I.D. Nos. 1 – 4119 and 4125 – 4377. In preferred embodiments, the present invention also provides nucleic acid molecule encoding such ribozymes; further preferably, the nucleic acid is DNA or cDNA. Even further preferably, the nucleic acid molecule is under the control of a promoter to transcribe the nucleic acid.

In another aspect, the present invention provides host cells containing the ribozymes described herein, vectors comprising the nucleic acid encoding the ribozymes described herein, and host cells comprising such a vector. Preferably, the vector is a plasmid, a virus, retrotransposon, a cosmid or a retrovirus. In one embodiment where the vector is a retroviral vector, the nucleic acid molecule encoding the ribozyme under the control of a promoter, which is preferably a pol III promoter, further preferably a human $tRNA^{Val}$ promoter or an adenovirus VA1 promoter, is inserted between the 5' and 3' long terminal repeat sequences of the retrovirus.

The present invention also provides a host cell stably transformed with such a retroviral vector. Preferably, the host cell is a murine or a human cell.

In a further aspect, the present invention provides methods for producing a ribozyme, the ribozyme being able to treat or prevent restenosis, which method comprises providing a nucleic acid molecule (*e.g.*, DNA) encoding the ribozyme under the transcriptional control of a promoter, and transcribing the nucleic acid molecule to produce the ribozyme. Preferably, the method further comprises purifying the ribozyme produced. The ribozyme may be produced *in vitro*, *in vivo* or *ex vivo*.

In yet another aspect, the present invention provides methods of treating or preventing restenosis, which method comprises introducing into the cell an effective amount of the ribozymes described herein. In one embodiment, such methods comprise introducing into the cell an effective amount of DNA encoding a ribozyme as described herein and transcribing the DNA to produce the ribozyme. Preferably, the cell is a human cell.

In still a further aspect, the present invention provides methods of treating or preventing restenosis are provided, which methods comprise introducing into the cell an effective amount of a nucleic acid molecule (*e.g.*, DNA) encoding a ribozyme as described herein and transcribing the DNA to produce the ribozyme. Preferably, the cell is a human cell.

In preferred embodiments, the methods further comprise administering the cell transduced with a retroviral vector to a mammal of the same species as that from which the transduced cell was obtained. In other preferred embodiments, the cell transduced with the retroviral vector has been obtained from the mammal receiving the transduced cell.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein that describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety as if each were individually noted for incorporation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of which shows the general structure of a chimeric DNA/RNA ribozyme (SEQ ID NOs: 4385 and 4386).

Figure 2 is a photograph of a gel which shows the stability of chimeric ribozymes PN30003, 30004, and 30005 in human vascular smooth muscle cell lysate.

Figure 3 is a photograph of a gel which shows the stability of chimeric ribozymes PN30003 and 30005 in serum.

Figure 4 is a schematic illustration of vector pLNT-Rz.

Figure 5 is a schematic illustration of a representative hairpin ribozyme (SEQ ID NOs: 4387 and 4388).

Figure 6 is a graph which illustrates the effects of ribozymes on a balloon injured rat carotid artery.

Figure 7 is a graph which illustrates the effects of ribozymes on a balloon injured rat carotid artery.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Ribozyme" refers to a nucleic acid molecule which is capable of cleaving a specific nucleic acid sequence. Ribozymes may be composed of RNA, DNA, nucleic acid analogues (*e.g.*, phosphorothioates), or any combination of these (*e.g.*, DNA/RNA chimerics). Within particularly preferred embodiments, a ribozyme should be understood to refer to RNA molecules that contain anti-sense sequences for specific recognition, and an RNA-cleaving enzymatic activity.

"Ribozyme gene" refers to a nucleic acid molecule (*e.g.*, DNA) consisting of the ribozyme sequence which, when transcribed into RNA, will yield the ribozyme.

"Vector" refers to an assembly which is capable of expressing a ribozyme of interest. The vector may be composed of either deoxyribonucleic acids ("DNA") or ribonucleic acids ("RNA"). Optionally, the vector may include a polyadenylation sequence, one or more restriction sites, as well as one or more selectable markers such as neomycin phosphotransferase, hygromycin phosphotransferase or puromycin-N-acetyl-transferase. Additionally, depending on the host cell chosen and the vector employed, other genetic elements such as an origin of replication, additional nucleic acid restriction sites, enhancers, sequences conferring inducibility of transcription, and selectable markers, may also be incorporated into the vectors described herein.

"Nucleic acid" or "nucleic acid molecule" refers to any of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acids can be composed of monomers that are naturally-occurring nucleotides (such as deoxyribonucleotides and ribonucleotides), or analogs of naturally-occurring nucleotides (*e.g.*, α-enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have modifications in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. The term "nucleic acid" also includes so-called "peptide nucleic acids," which comprise naturally-occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded.

"Isolated nucleic acid molecule" is a nucleic acid molecule that is not integrated in the genomic DNA of an organism. For example, a DNA molecule that encodes a gene that has been separated from the genomic DNA of a eukaryotic cell is an isolated DNA molecule. Another example of an isolated nucleic acid molecule is a chemically-synthesized nucleic acid molecule that is not integrated in the genome of an organism.

"Promoter" is a nucleotide sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5' region of a gene, proximal to the transcriptional start site of a structural gene. If a promoter is an inducible promoter,

then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Restenosis is a major clinical problem and as the result of a need for repeat hospitalization, repeat angioplasty or bypass surgery, restenosis costs the nation's health care system in excess of $1 billion per year. Restenosis is believed to comprise three important components. First, myointimal proliferation of vascular smooth muscle cells and the subsequent deposition of ECM results in a fibrocellular mass which can encroach upon the vascular lumen. Second, following acute angioplasty, there may be significant elastic recoil of the artery which contributes to a late loss of luminal dimension. Finally, platelets and thrombus adherent to the vascular wall may, over time, organize into a fibrocellular mass.

As discussed in more detail below, by interfering with cell-cycle control of cells which might otherwise proliferate following vascular injury, restenosis can be effectively treated and/or prevented. This invention accomplishes such by providing ribozymes and methods of using ribozymes that directly block cell cycle control following vascular injury. Representative examples of suitable ribozyme targets include cdk1 ribozyme binding sites (SEQ ID NOS: 1-149); cdk2 ribozyme binding sites (SEQ ID NOS: 150-3010); cdk3 ribozyme binding sites (SEQ ID NOS: 302-405); cdk4 ribozyme binding sites (SEQ ID NOS: 406-526); cdk6 ribozyme binding sites (SEQ ID NOS: 527-665); cdk7 ribozyme binding sites (SEQ ID NOS: 666-866); cdk8 ribozyme binding sites (SEQ ID NOS: 867-1112); cdk-we-hu ribozyme binding sites (SEQ ID NOS: 1113-1408); cyclin A2 ribozyme binding sites (SEQ ID NOS: 1409-1614); cyclin C ribozyme binding sites (SEQ ID NOS: 1615-1819); cyclin D1 ribozyme binding sites (SEQ ID NOS: 1820-1889); cyclin D2 ribozyme binding sites (SEQ ID NOS: 1890-1975); cyclin D3 ribozyme binding sites (SEQ ID NOS: 1976-2053); cyclin E ribozyme binding sites (SEQ ID NOS: 2054-2318); cyclin F ribozyme binding sites (SEQ ID NOS: 2319-2561); cyclin G1 ribozyme binding sites (SEQ ID NOS: 2562-2787); cyclin H ribozyme binding sites (SEQ ID NOS: 2788-2964); cyclin A1 ribozyme binding sites (SEQ ID NOS: 2965-3257); cyclin B1 ribozyme binding sites (SEQ ID

NOS: 3258-3478); cdc25 hs ribozyme binding sites (SEQ ID NOS: 3479-3854); PCBA HH ribozyme binding sites (SEQ ID NOS: 3855-4115); and chimeric hairpin ribozymes: SEQ ID NOS: 4116-4119).

RIBOZYMES

As noted above, the present invention provides ribozymes having the ability to cleave or otherwise inhibit nucleic acid molecules which are either directly, or indirectly (e.g., they encode proteins) involved in cell-cycle control (e.g. recognition sites of Sequence I.D. Nos. 1 - 4119 and 4125 - 4377. Several different types of ribozymes may be constructed for use within the present invention, including for example, hammerhead ribozymes (Rossi, J.J. et al., *Pharmac. Ther. 50*:245-254, 1991) (Forster and Symons, *Cell 48*:211-220, 1987; Haseloff and Gerlach, *Nature 328*:596-600, 1988; Walbot and Bruening, *Nature 334*:196, 1988; Haseloff and Gerlach, *Nature 334*:585, 1988; Haseloff et al., U.S. Patent No. 5,254,678), hairpin ribozymes (Hampel et al., *Nucl. Acids Res. 18*:299-304, 1990, and U.S. Patent No. 5,254,678), hepatitis delta virus ribozymes (Perrotta and Been, *Biochem. 31*:16, 1992), Group I intron ribozymes (Cech et al., U.S. Patent No. 4,987,071) and RNase P ribozymes (Takada et al., *Cell 35*:849, 1983); (*see* also, WO 95/29241, entitled "Ribozymes with Product Ejection by Strand Displacement"; and WO 95/31551, entitled "Novel Enzymatic RNA Molecules."

Cech et al. (U.S. Patent No. 4,987,071, issued January 22, 1991) has disclosed the preparation and use of ribozymes which are based on the properties of the *Tetrahymena* ribosomal RNA self-splicing reaction. These ribozymes require an eight base pair target site and free guanosine (or guanosine derivatives). A temperature optimum of 50°C is reported for the endoribonuclease activity. The fragments that arise from cleavage contain 5'-phosphate and 3'-hydroxyl groups and a free guanosine nucleotide added to the 5'-end of the cleaved RNA.

In contrast to the ribozymes of Cech et al., particularly preferred ribozymes of the present invention hybridize efficiently to target sequences at physiological temperatures, making them suitable for use *in vivo*, and not merely as

research tools (*see* column 15, lines 18 to 42, of Cech et al., U.S. Patent No. 4,987,071). Thus, particularly preferred ribozymes for use within the present invention include hairpin ribozymes (for example, as described by Hampel et al., European Patent Publication No. 0 360 257, published March 26, 1990) and hammerhead ribozymes. Briefly, the sequence requirement for the hairpin ribozyme is any RNA sequence consisting of NNNBN*GUC$(N)_x$ (Sequence ID Nos. 4120-4124) (where x is any number from 6 to 10, N*G is the cleavage site, B is any of G, C, or U, and N is any of G, U, C, or A). Representative examples of recognition or target sequences for hairpin ribozymes are set forth below in the Examples. Additionally, the backbone or common region of the hairpin ribozyme can be designed using the nucleotide sequence of the native hairpin ribozyme (Hampel et al., *Nucl. Acids Res. 18*:299-304, 1990) or it can be modified to include a "tetraloop" structure that increases stability and catalytic activity (see Example 2; see also Yu et al., *Virology 206*:381-386, 1995; Cheong et al., *Nature 346*:680-682, 1990; Anderson et al., *Nucl. Acids Res. 22*:1096-1100, 1994).

The sequence requirement at the cleavage site for the hammerhead ribozyme is any RNA sequence consisting of NUH (where N is any of G, U, C, or A and H represents C, U, or A) can be targeted. Accordingly, the same target within the hairpin leader sequence, GUC, is useful for the hammerhead ribozyme. The additional nucleotides of the hammerhead ribozyme or hairpin ribozyme is determined by the target flanking nucleotides and the hammerhead consensus sequence (*see* Ruffner et al., *Biochemistry 29*:10695-10702, 1990). This information, along with the sequences and disclosure provided herein, enables the production of hairpin ribozymes of this invention.

The ribozymes of this invention, as well as DNA encoding such ribozymes and other suitable nucleic acid molecules, described in more detail below, can be chemically synthesized using methods well known in the art for the synthesis of nucleic acid molecules (*see e.g.*, Heidenreich et al., *J. FASEB 70*(1):90-6, 1993; Sproat, *Curr. Opin. Biotechnol. 4*(1):20-28, 1993). Alternatively, commercial suppliers such as Promega, Madison, Wis., USA, provide a series of protocols suitable for the production of nucleic acid molecules such as ribozymes.

Within one aspect of the present invention, ribozymes are prepared from a DNA molecule or other nucleic acid molecule (which, upon transcription, yields an RNA molecule) operably linked to an RNA polymerase promoter, *e.g.*, the promoter for T7 RNA polymerase or SP6 RNA polymerase. Accordingly, also provided by this invention are nucleic acid molecules, *e.g.*, DNA or cDNA, coding for the ribozymes of this invention. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced *in vitro* upon incubation with the RNA polymerase and appropriate nucleotides. In a separate embodiment, the DNA may be inserted into an expression cassette, such as described in Cotten and Birnstiel, *EMBO J.* 8(12):3861-3866, 1989, and in Hempel et al., *Biochemistry* 28:4929-4933, 1989. A more detailed discussion of molecular biology methodology is disclosed in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989.

During synthesis, the ribozyme can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to RNase (Rossi et al., *Pharmac. Ther.* 50:245-254, 1991). Alternatively, the ribozyme can be modified to a phosphothio-analog for use in liposome delivery systems. This modification also renders the ribozyme resistant to endonuclease activity.

VECTORS

Use of ribozymes to treat restenosis involves introduction of functional ribozyme to the infected cell of interest. This can be accomplished by either synthesizing functional ribozyme *in vitro* prior to delivery, or, by delivery of DNA capable of driving ribozyme synthesis *in vivo*.

More specifically, within other aspects of the invention the ribozyme gene may be constructed within a vector which is suitable for introduction to a host cell (*e.g.*, prokaryotic or eukaryotic cells in culture or in the cells of an organism). Appropriate prokaryotic and eukaryotic cells can be transfected with an appropriate transfer vector containing the nucleic acid molecule encoding a ribozyme of this invention.

To produce the ribozymes with a vector *in vivo*, nucleotide sequences coding for ribozymes are preferably placed under the control of a eukaryotic promoter such as pol III (*e.g.*, tRNA or VA-1 from adenovirus), CMV, SV40 late, or SV40 early promoters. Within certain embodiments, the promoter may be a tissue or cell-specific promoter. Ribozymes may thus be produced directly from the transfer vector *in vivo*.

A wide variety of vectors may be utilized within the context of the present invention, including for example, plasmids, viruses, retrotransposons and cosmids. Representative examples include adenoviral vectors (*e.g.*, WO 94/26914, WO 93/9191; Yei et al., *Gene Therapy 1*:192-200, 1994; Kolls et al., *PNAS 91*(1):215-219, 1994; Kass-Eisler et al., *PNAS 90*(24):11498-502, 1993; Guzman et al., *Circulation 88*(6):2838-48, 1993; Guzman et al., *Cir. Res. 73*(6):1202-1207, 1993; Zabner et al., *Cell 75*(2):207-216, 1993; Li et al., *Hum Gene Ther. 4*(4):403-409, 1993; Caillaud et al., *Eur. J. Neurosci. 5*(10):1287-1291, 1993), adeno-associated type 1 ("AAV-1") or adeno-associated type 2 ("AAV-2") vectors (*see* WO 95/13365; Flotte et al., *PNAS 90*(22):10613-10617, 1993), hepatitis delta vectors, live, attenuated delta viruses and herpes viral vectors (*e.g.*, U.S. Patent No. 5,288,641), as well as vectors which are disclosed within U.S. Patent No. 5,166,320. Other representative vectors include retroviral vectors (*e.g.*, EP 0 415 731; WO 90/07936; WO 91/02805; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218). General methods of using such vectors in gene therapy are well known in the art, see, for example, Larrick, J.W. and Burck, K.L., *Gene Therapy: Application of Molecular Biology*, Elsevier Science Publishing Co., Inc., New York, New York, 1991 and Kreigler, M., *Gene Transfer and Expression: A Laboratory Manual*, W.H. Freeman and Company, New York, 1990.

Further provided by this invention are vectors having more than one nucleic acid molecule encoding a ribozyme of this invention, each molecule under the control of a separate eukaryotic promoter (or, an Internal Ribosome Entry Site or "IRES") or alternatively, under the control of single eukaryotic promoter. Representative examples of other nucleic acid molecules which may be delivered by the vectors of the present invention include therapeutic molecules such as interferon (*e.g.*,

alpha, beta or gamma), as well as a wide variety of other cytokines or growth factors, and facilitators which assist or aid ribozymes in cleaving a target sequence by unwinding or otherwise limiting secondary folding which might otherwise inhibit the ribozyme (see Example 4). These vectors provide the advantage of providing multi-functional therapy against Restenosis, preferably with the various therapies working together in synergy.

Host prokaryotic and eukaryotic cells stably harboring the vectors described above also are provided by this invention. Suitable host cells include bacterial cells, rat cells, mouse cells, and human cells.

DELIVERY

Within certain aspects of the invention, ribozyme molecules, or nucleic acid molecules which encode the ribozyme, may be introduced into a host cell utilizing a vehicle, or by various physical methods. Representative examples of such methods include transformation using calcium phosphate precipitation (Dubensky et al., *PNAS 81*:7529-7533, 1984), direct microinjection of such nucleic acid molecules into intact target cells (Acsadi et al., *Nature 352*:815-818, 1991), and electroporation whereby cells suspended in a conducting solution are subjected to an intense electric field in order to transiently polarize the membrane, allowing entry of the nucleic acid molecules. Other procedures include the use of nucleic acid molecules linked to an inactive adenovirus (Cotton et al., *PNAS 89*:6094, 1990), lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), polycation compounds such as polylysine, receptor specific ligands, liposomes entrapping the nucleic acid molecules, spheroplast fusion whereby *E. coli* containing the nucleic acid molecules are stripped of their outer cell walls and fused to animal cells using polyethylene glycol, viral transduction, (Cline et al., *Pharmac. Ther. 29*:69, 1985; and Friedmann et al., *Science 244*:1275, 1989), and DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989). In one embodiment, the ribozyme is introduced into the host cell using a liposome.

Within further embodiments of the invention, additional therapeutic molecules (*e.g.*, interferon) or facilitators may be delivered utilizing the methods described herein. Such delivery may be either simultaneous to, or before or after the delivery of a ribozyme or vector expressing ribozymes.

PHARMACEUTICAL COMPOSITIONS

As noted above, pharmaceutical compositions (or "medicaments") also are provided by this invention. These compositions contain any of the above described ribozymes, DNA molecules, vectors or host cells, along with a pharmaceutically or physiologically acceptable carrier, excipient, or, diluent. Generally, such carriers should be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents. Particularly preferred carriers include cholesterols such as DOTAP:cholesterol.

Pharmaceutical compositions of the present invention may also be prepared to contain, or express (*e.g.*, if a vector), one or more additional therapeutic molecules (*e.g.*, interferon) or facilitators.

In addition, the pharmaceutical compositions of the present invention may be prepared for administration by a variety of different routes, including for example, intravenously (*e.g.*, into a vein by balloon catheter), or [on the outside of the vein]. In addition, pharmaceutical compositions of the present invention may be placed within containers, along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (*e.g.*, water, saline or PBS) which may be necessary to reconstitute the pharmaceutical composition

Pharmaceutical compositions are useful for both diagnostic or therapeutic purposes.

THERAPEUTIC METHODS

Methods of interfering with or preventing restenosis are also provided by this invention. More specifically, within one aspect of the present invention restenosis may be treated or prevented by administering to a warm-blooded animal (e.g., a human) a therapeutically effective amount of ribozyme, and/or, nucleic acid molecule or vector which encodes the ribozyme. Generally, such methods may be utilized to treat restenosis in vascular tissue; however, other tissues where stenosis is a problem may similarly be treated.

Such methods require contacting desired cells with an effective amount of ribozyme of this invention or, alternatively, by transducing the cell with an effective amount of vector having a nucleic acid molecule encoding the ribozyme. A suitable "therapeutically effective amount" will depend on the nature and extent of diseased tissue being treated, or, if a medical procedure is contemplated in which restenosis can be expected, prevented. Such "therapeutically effective amounts" can be readily determined by those of skill in the art using well known methodology, and suitable animal models (e.g. a rat or porcine model), or, based upon clinical trials. As utilized herein, a patient is deemed "treated" if restenosis is reversed or inhibited within a patient in a quantifiable manner. Similarly, a patient restenosis is deemed "prevented" if the likelihood of, or, occurrence of restenosis due to either disease or a medical or surgical intervention (e.g., balloon angioplasty, or, delivery of stent) decreases in a statistically significant manner.

When exogenously delivering the ribozyme, the RNA molecule can be embedded within a stable RNA molecule or in another form of protective environment, such as a liposome. Alternatively, the RNA can be embedded within RNase-resistant DNA counterparts. Cellular uptake of the exogenous ribozyme can be enhanced by attaching chemical groups to the DNA ends, such as cholesteryl moieties (Letsinger et al., *P.N.A.S., U.S.A.*, 1989).

In another aspect of the invention, the target cell is transduced under conditions favoring insertion of the vector into the target cell and stable expression of the nucleic acid encoding the ribozyme. The target cell can include but is not limited to vascular smooth muscle cells.

Ribozymes, ribozyme genes, and vectors encoding such genes may readily be delivered to a desired site by a variety of methods, including for example, by balloon catheter, by stent, or by microinjection (see, e.g., U.S. Patent Nos. 5,840,064, 5,836,905 and 5,833,659). Further, the ribozyme, gene, or vector may be delivered transluminally, within the smooth muscle cells of the lumen, or exoluminally. In addition, the ribozyme, ribozyme gene or vector may be readily incorporated into a biodegradable polymer, sphere, pleuroinc gel, or the like to aid incorporation into cells.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

CRITERIA FOR RIBOZYME SITE SELECTION

A. Selection of Sites for Hairpin Ribozymes

Hairpin ribozymes suitable for use within the present invention preferably recognize the following sequence of RNA: NNNBNGUCNNNNNNNN (SEQ ID NO:4122) wherein the ribozyme is constructed so as to be complementary to the underlined sequences, and wherein B is C, G or U. The sequence GUC must be conserved for all hairpin ribozymes described below. Other nucleotides ("N" as underlined above) preferably have a high degree of sequence conservation in order to limit the need for multiple ribozymes against the same target site. Representative GUC hairpin ribozyme recognition sites for various genes are provided below in Tables 1-4.

Table 1

Hairpin Ribozyme Recognition Sites for cdc 2 kinase

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
175	ACTTCGTCATCCAAAT	4125
189	ATATAGTCAGTCTTCA	4126
193	AGTCAGTCTTCAGGAT	4127
289	TCCTGGTCAGTACATG	4128
355	GTTTTGTCACTCTAGA	4129
530	CTGGGGTCAGCTCGTT	4130

Table 2

Hairpin Ribozyme Recognition Sites for Cyclin B1

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
12	TCCGAGTCACCAGGAA	4131
281	CCAGTGTCTGAGCCAG	4132
427	CCTGTGTCAGGCTTTC	4133
558	AAGCAGTCAGACCAAA	4134
580	ACTGGGTCGGGAAGTC	4135
678	TGACTGTCTCCATTAT	4136
	TTGGTGTCACTGCCAT	4137
	CTTTGGTCTGGGTCGG	4138
	TCTGGGTCGGCCTCTA	4139
	TACCTGTCATATACTG	4140
	ATGTAGTCATGGTAAA	4141
	TGACTGTCAAGAACAA	4142

Table 3

Hairpin Ribozyme Recognition Sites for PCNA

UCL. POS.	SEQUENCE (5' to 3')	I.D. No.
	GAGTGGTCGTTGTCTT	4143
	TCGTTGTCTTTCTAGG	4144
18	GCCTGGTCCAGGGCTC	4145
125	GACTCGTCCCACGTCT	4146
158	CTGCGGTCTGAGGGCT	4147
	AAATTGTCACAGACAA	4148
867	TTTCTGTCACCAAATT	4149
	ATCTGGTCTAGTTAAC	4150
	TTTTTGTCTCTTAGAA	4151
	AAAGGGTCTTGACTCT	4152

Table 4

Hairpin Ribozyme Recognition Sites for Lysyl Oxidase

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
225	CCGCCGTCCCTGGTGC	4153
333	CTGGAGTCACCGCTGG	4154
364	CGCCCGTCACTGGTTC	4155
631	GTACGGTCTCCCAGAC	4156
671	CAGGCGTCCACGTACG	4157
730	AAACTGTCTGGCCAGT	4158
970	TTTCTGTCTTGAAGAC	4159

B. Selection of Cleavage Sites for Hammerhead Ribozymes

Hammerhead ribozymes suitable for use within the present invention preferably recognize the sequence NUH, wherein N is any of G, U, C, or A and H is C, U, or A. Representative hammerhead target sites include:

Table 5

Hammerhead Ribozyme Recognition Sites for cdc 2 kinase

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
81	TACAGGTCAAGTGGTA	4160
159	AAATTTCTCTATTAAAG	4161
195	AGTCAGTCTTCAGGAT	4162
532	CTGGGGTCAGCTCGTT	4163
	CGCGGAATAATAAGCCGG	4164
	GGAATAATAAGCCGGGAT	4165
	GCCGGGATCTACCATACC	4166
	CGGGATCTACCATACCAT	4167
	TCTACCATACCATTGACT	4168
	CATACCATTGACTAACTA	4169
	CATTGACTAACTATGGAA	4170
	GACTAACTATGGAAGATT	4171

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
	TGGAAGATTATACCAAAA	4172
	GGAAGATTATACCAAAAT	4173
	AAGATTATACCAAAATAG	4174
	ACCAAAATAGAGAAAATT	4175
	GAGAAAATTGGAGAAGGT	4176
	GAGAAGGTACCTATGGAG	4177
	AGGTACCTATGGAGTTGTG	4178
	TATGGAGTTGTGTATAAG	4179
	AGTTGTGTATAAGGGTAG	4180
	TTGTGTATAAGGGTAGAC	4181
	ATAAGGGTAGACACAAAA	4182
	ACAAAACTACAGGTCAAG	4183
	CTACAGGTCAAGTGGTAG	4184
	CAAGTGGTAGCCATGAAA	4185
	AAAAAAATCAGACTAGAA	4186
	ATCAGACTAGAAAGTGAA	4187
	GAAGGGGTTCCTAGTACT	4188
	AAGGGGTTCCTAGTACTG	4189
	GGGTTCCTAGTACTGCAA	4190
	TTCCTAGTACTGCAATTC	4191
	ACTGCAATTCGGGAAATT	4192
	CTGCAATTCGGGAAATTT	4193
	CGGGAAATTTCTCTATTA	4194
	GGGAAATTTCTCTATTAA	4195
	GGAAATTTCTCTATTAAA	4196
	AAATTTCTCTATTAAAGG	4197
	ATTTCTCTATTAAAGGAA	4198
	TTCTCTATTAAAGGAACT	4199
	TCTCTATTAAAGGAACTT	4200
	AAGGAACTTCGTCATCCA	4201
	AGGAACTTCGTCATCCAA	4202
	AACTTCGTCATCCAAATA	4203

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
	TTCGTCATCCAAATATAG	4204
	ATCCAAATATAGTCAGTC	4205
	CCAAATATAGTCAGTCTT	4206
	AATATAGTCAGTCTTCAG	4207
	TAGTCAGTCTTCAGGATG	4208
	GTCAGTCTTCAGGATGTG	4209
	TCAGTCTTCAGGATGTGC	4210
	GATGTGCTTATGCAGGATT	4211
	ATGTGCTTATGCAGGATTC	4212
	TGCAGGATTCCAGGTTAT	4213
	GCAGGATTCCAGGTTATA	4214
	TTCCAGGTTATATCTCAT	4215
	TCCAGGTTATATCTCATC	4216
	CAGGTTATATCTCATCTT	4217
	GGTTATATCTCATCTTTG	4218
	TTATATCTCATCTTTGAG	4219
	TATCTCATCTTTGAGTTT	4220
	TCTCATCTTTGAGTTTCT	4221
	CTCATCTTTGAGTTTCTT	4222
	CTTTGAGTTTCTTTCCAT	4223
	TTTGAGTTTCTTTCCATG	4224
	TTGAGTTTCTTTCCATGG	4225
	GAGTTTCTTTCCATGGAT	4226
	AGTTTCTTTCCATGGATC	4227
	CCATGGATCTGAAGAAAT	4228
	GAAGAAATACTTGGATTC	4229
	GAAATACTTGGATTCTAT	4230
	ACTTGGATTCTATCCCTC	4231
	CTTGGATTCTATCCCTCC	4232
	TGGATTCTATCCCTCCTG	4233
	GATTCTATCCCTCCTGGT	4234
	CTATCCCTCCTGGTCAGT	4235

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
	CTCCTGGTCAGTACATGG	4236
	TGGTCAGTACATGGATTC	4237
	ACATGGATTCTTCACTTG	4238
	CATGGATTCTTCACTTGT	4239
	TGGATTCTTCACTTGTTA	4240
	GGATTCTTCACTTGTTAA	4241
	TCTTCACTTGTTAAGAGT	4242
	TCACTTGTTAAGAGTTAT	4243
	CACTTGTTAAGAGTTATT	4244
	TTAAGAGTTATTTATACC	4245
	TAAGAGTTATTTATACCA	4246
	AGAGTTATTTATACCAAA	4247
	GAGTTATTTATACCAAAT	4248
	AGTTATTTATACCAAATC	4249
	TTATTTATACCAAATCCT	4250
	CAAATCCTACAGGGGATT	4251
	CAGGGGATTGTGTTTTGT	4252
	GATTGTGTTTTGTCACTC	4253
	ATTGTGTTTTGTCACTCT	4254
	TTGTGTTTTGTCACTCTA	4255
	TGTTTTGTCACTCTAGAA	4256
	TTGTCACTCTAGAAGAGT	4257
	GTCACTCTAGAAGAGTTC	4258
	AGAAGAGTTCTTCACAGA	4259
	GAAGAGTTCTTCACAGAG	4260
	AGAGTTCTTCACAGAGAC	4261
	CAGAGACTTAAAACCTCA	4262
	AGAGACTTAAAACCTCAA	4263
	TAAAACCTCAAAATCTCT	4264
	CTCAAAATCTCTTGATTG	4265
	CAAAATCTCTTGATTGAT	4266
	AAATCTCTTGATTGATGA	4267

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
	CTCTTGATTGATGACAAA	4268
	GGAACAATTAAACTGGCT	4269
	TGGCTGATTTTGGCCTTG	4270
	GGCTGATTTTGGCCTTGC	4271
	GCTGATTTTGGCCTTGCC	4272
	TTTGGCCTTGCCAGAGCT	4273
	CCAGAGCTTTTGGAATAC	4274
	CAGAGCTTTTGGAATACC	4275
	AGAGCTTTTGGAATACCT	4276
	TTTGGAATACCTATCAGA	4277
	GAATACCTATCAGAGTAT	4278
	ATACCTATCAGAGTATAT	4279
	ATCAGAGTATATACACAT	4280
	CAGAGTATATACACATGA	4281
	GAGTATATACACATGAGG	4282
	CATGAGGTAGTAACACTC	4283
	GAGGTAGTAACACTCTGG	4284
	ACTCTGGTACAGATCTCC	4285
	GTACAGATCTCCAGAAGT	4286
	ACAGATCTCCAGAAGTAT	4287
	CCAGAAGTATTGCTGGGG	4288
	AGAAGTATTGCTGGGGTC	4289
	GCTGGGGTCAGCTCGTTA	4290
	GGTCAGCTCGTTACTCAA	4291
	CAGCTCGTTACTCAACTC	4292
	AGCTCGTTACTCAACTCC	4293
	TCGTTACTCAACTCCAGT	4294
	ACTCAACTCCAGTTGACA	4295
	ACTCCAGTTGACATTTGG	4296
	GTTGACATTTGGAGTATA	4297
	TTGACATTTGGAGTATAG	4298
	TTTGGAGTATAGGCACCA	4299

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
	TGGAGTATAGGCACCATA	4300
	GGCACCATATTTGCTGAA	4301
	CACCATATTTGCTGAACT	4302
	ACCATATTTGCTGAACTA	4303
	GCTGAACTAGCAACTAAG	4304
	TAGCAACTAAGAAACCAT	4305
	GAAACCATTTTCCATGGG	4306
	AAACCATTTTCCATGGGG	4307
	AACCATTTTCCATGGGGA	4308
	ACCATTTTCCATGGGGAT	4309
	ATGGGGATTCAGAAATTG	4310
	TGGGGATTCAGAAATTGA	4311
	AAATTGATCAACTCTTCA	4312
	GATCAACTCTTCAGGATT	4313
	TCAACTCTTCAGGATTTT	4314
	TTCAGGATTTTCAGAGCT	4315
	TCAGGATTTTCAGAGCTT	4316
	CAGGATTTTCAGAGCTTT	4317
	AGGATTTTCAGAGCTTTG	4318
	TCAGAGCTTTGGGCACTC	4319
	CAGAGCTTTGGGCACTCC	4320
	TGGGCACTCCCAATAATG	4321
	CTCCCAATAATGAAGTGT	4322
	AGTGGAATCTTTACAGGA	4323
	TGGAATCTTTACAGGACT	4324
	GGAATCTTTACAGGACTA	4325
	GAATCTTTACAGGACTAT	4326
	ACAGGACTATAAGAATAC	4327
	AGGACTATAAGAATACAT	4328
	ATAAGAATACATTTCCCA	4329
	GAATACATTTCCCAAATG	4330
	AATACATTTCCCAAATGG	4331

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
	ATACATTTCCCAAATGGA	4332
	GGAAGCCTAGCATCCCAT	4333
	CCTAGCATCCCATGTCAA	4334
	TCCCATGTCAAAAACTTG	4335
	CAAAAACTTGGATGAAAA	4336
	AAATGGCTTGGATTTGCT	4337
	GCTTGGATTTGCTCTCGA	4338
	CTTGGATTTGCTCTCGAA	4339
	GATTTGCTCTCGAAAATG	4340
	TTTGCTCTCGAAAATGTT	4341
	GAAAATGTTAATCTATGA	4342
	AAAATGTTAATCTATGAT	4343
	ATGTTAATCTATGATCCA	4344
	GTTAATCTATGATCCAGC	4345
	TCTATGATCCAGCCAAAC	4346
	AAACGAATTTCTGGCAAA	4347
	AACGAATTTCTGGCAAAA	4348
	ACGAATTTCTGGCAAAAT	4349
	CACTGAATCATCCATATT	4350
	TGAATCATCCATATTTTA	4351
	TCATCCATATTTTAATGA	4352
	ATCCATATTTTAATGATT	4353
	TCCATATTTTAATGATTT	4354
	CCATATTTTAATGATTTG	4355
	CATATTTTAATGATTTGG	4356
	TTAATGATTTGGACAATC	4357
	TAATGATTTGGACAATCA	4358
	TGGACAATCAGATTAAGA	4359
	GAAGATGTAGCTTTCTGA	4360

Table 6

Hammerhead Ribozyme Recognition Sites for Cyclin B1

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
14	TCCGAGTCACCAGGAA	4361
283	CCAGTGTCTGAGCCAG	4362
429	CCTGTGTCAGGCTTTC	4363
560	AAGCAGTCAGACCAAA	4364
582	ACTGGGTCGGGAAGTC	4365
680	TGACTGTCTCCATTAT	4366

Table 7

Hammerhead Ribozyme Recognition Sites for PCNA

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
20	GCCTGGTCCAGGGCTC	4367
127	GACTCGTCCCACGTCT	4368
160	CTGCGGTCTGAGGGCT	4369
869	TTTCTGTCACCAAATT	4370

Table 8

Hammerhead Ribozyme Recognition Sites for Lysyl Oxidase

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
227	CCGCCGTCCCTGGTGC	4371
335	CTGGAGTCACCGCTGG	4372
366	CGCCCGTCACTGGTTC	4373
633	GTACGGTCTCCCAGAC	4374
673	CAGGCGTCCACGTACG	4375
732	AAACTGTCTGGCCAGT	4376
972	TTTCTGTCTTGAAGAC	4377

Table 9

Further Ribozyme Recognition Sites

TARGET SITE	I.D. No.
cdk1 ribozyme binding sites:	1-149
cdk2 ribozyme binding sites:	150-301
cdk3 ribozyme binding sites:	302-405
cdk4 ribozyme binding sites:	406-526
cdk6 ribozyme binding sites:	527-665
cdk7 ribozyme binding sites:	666-866
cdk8 ribozyme binding sites:	867-1112
cdk-we-hu ribozyme binding sites:	1113-1408
cyclin A2 ribozyme binding sites:	1409-1614
cyclin C ribozyme binding sites:	1615-1819
cyclin D1 ribozyme binding sites:	1820-1889
cyclin D2 ribozyme binding sites:	1890-1975
cyclin D3 ribozyme binding sites:	1976-2053
cyclin E ribozyme binding sites:	2054-2318
cyclin F ribozyme binding sites:	2319-2561
cyclin G1 ribozyme binding sites:	2562-2787
cyclin H ribozyme binding sites:	2788-2964
cyclin A1 ribozyme binding sites:	2965-3257
cyclin B1 ribozyme binding sites:	3258-3478
cdc25 hs ribozyme binding sites:	3479-3854
PCBA HH ribozyme binding sites:	3855-4115
Example chimeric hairpin ribozymes:	4116-4119

EXAMPLE 2

CONSTRUCTION OF HAIRPIN RIBOZYMES

Two single-stranded DNA oligonucleotides are chemically synthesized such that, when combined and converted into double-stranded DNA, they contain the entire hairpin ribozyme, including nucleotides complementary to the target site. In addition, restriction enzyme recognition sites may be placed on either end to facilitate subsequent cloning. More specifically, the oligonucleotides are hybridized together and converted to double-stranded DNA using either Klenow DNA polymerase or Taq DNA polymerase. The resulting DNA is cleaved with restriction enzymes *Bam*HI and *Mlu*I, purified and cloned into vectors for *in vitro* transcription (pGEM, ProMega, Madison, Wis.) or for retrovirus production and mammalian expression (pLNL/MJT backbone). Representative hairpin ribozymes are set forth below (note that the underlined sequences indicate the sites wherein the ribozyme binds the target sequence):

cdc-2 530 (Sequence I.D. No. 4378)
5' AACGAGCTAGAACCAGACCAGAGAAACACACGTTGTGGTATATTACCTGGTA 3'

Cyclin B1 281 (Sequence I.D. No. 4379)
5' CTGGCTCAAGAACTGGACCAGAGAAACACACGTTGTGGTATATTACCTGGTA 3'

Lysyl Oxidase 333 (Sequence I.D. No. 4380)
5' CCAGCGGTAGAACCAGACCAGAGAAACACACGTTGTGGTATATTACCTGGTA 3'

PCNA 158 (Sequence I.D. No. 4381)
5' AGCCCTCAAGAAGCAGACCAGAGAAACACACGTTGTGGTATATTACCTGGTA 3'

Defective ribozymes for use as controls may be constructed as described above, with the exception that the sequence AAA is changed to a UGC as shown in Figure 2.

EXAMPLE 3

CONSTRUCTION OF HAMMERHEAD RIBOZYMES

Chimeric hammerhead ribozymes (i.e., RNA/DNA hybrids) are designed to have an appropriate NUH sequence for ribozyme cleavage. Ribozymes are chemically synthesized with the general structure shown in figure 1. The binding arms bases and stem loop comprise DNA, and the catalytic domain comprises RNA and/or 2'O methyl RNA bases. Specific examples of synthetic human hammerhead ribozymes targeting PCNA are shown below (DNA bases shown in upper case, RNA bases as lower case, and 2' O methyl RNA as lower case italics):

Sequence ID No. 4382: PN30003 PCN1-HH Length: 40
5' GAGCCCTG cugaugag CAATTTTTTG cgaaa ACCAGGCGC 3'

Sequence ID No. 4383: PN30004 OptPCN1-ome HH Length: 38
5' AGCCC *ug* cuga *u* g *agg* CCGTAAGG *cc* ga *a* a *cc* AGGCGC 3'

Sequence ID No. 4384: PN30005 StabPCN1-ome HH Length: 38
5' AGCCC *ugcu* ga *u* g *agg* CCGTAAGG *cc* ga *a* a *cc* AGGCGC 3'

Alteration of the base composition at the stem loop and catalytic domain increases the catalytic activity of the chimeric ribozyme as assayed by in vitro cleavage (EXAMPLE 5). The substitution of 2' O methyl bases for RNA bases enhances the stability of the chimeric ribozymes in human vascular smooth muscle cell lysate, and in serum. The assay consists of incubating 10 μg of ribozyme with 100 μl of human vascular smooth muscle cell lysate at 37°C for times ranging from 30 seconds to 240 minutes, then separating the intact ribozyme from degradation products on a 15% PAGE, staining with SYBRgreen (Molecular Probes, Eugene, OR), and quantifying by phosphorimager analysis (Molecular Dynamics).

By making specific base modifications to the structure of the ribozymes, the half-life in cell lysate was increased sequentially from approximately 2.5 hours for PN30003, to 3.5 hours for PN30004, and to greater than 10 hours for PN30005 (figure 2). In serum, the half-life of PN30003 is less than 30 seconds. Specific base

modifications to ribozyme PN30005 increased the half-life in serum to greater than 4 hours (figure 3).

A scrambled sequence polynucleotide including the same composition of ribonucleotides and deoxyribonucleotides is also synthesized for each ribozyme to serve as a control with no catalytic activity. Lipofectin may be utilized to enhance the uptake of ribozyme into the cells.

EXAMPLE 4

CONSTRUCTION OF RIBOZYME MAMMALIAN EXPRESSION VECTORS

Plasmid pMJT (Yu et al., *Proc. Nat'l Acad. Sci. USA 90*:6340-6344, 1993), which contains the anti-U5 HIV ribozyme driven by the $tRNA^{val}$ RNA pol III promoter, is digested with *Bam*HI and *Mlu*I, and the vector purified from the ribozyme fragment. The hairpin ribozymes, as described above, are excised from the pGem vector with *Bam*HI and *Mlu*I, purified, and ligated into the empty pMJT vector. The resulting vector is designated pLNT-Rz (*see* Figure 4, and contains the Moloney LTR driving the neomycin resistance gene and the $tRNA^{val}$ RNA pol III promoter driving expression of the ribozyme.

EXAMPLE 5

IN VITRO CLEAVAGE ASSAYS

Hairpin or hammerhead ribozymes are tested for cleavage activity in an in vitro assay. Ribozyme and substrate synthesis is achieved by a new method of plasmid-independent in vitro transcription (Welch et al 1997). Briefly, oligonucleotides are synthesized (Retrogen, San Diego CA) with the T7 RNA polymerase promoter sequence contiguous with the ribozyme or substrate sequences, to allow for in vitro transcription of annealed oligonucleotides without the need for plasmid cloning. In vitro cleavage is tested in two hour time course reactions in 40 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 mM spermidine, at 37°C (Welch et al 1997). Reaction products are analyzed

by polyacrylamide gel electrophoresis (PAGE) and quantified by phosphorimager analysis (Molecular Dynamics). The Michaelis constant (K_m^{app}) and k_2 are determined for each ribozyme by performing single turnover kinetic experiments with ribozyme concentrations of 2-4 nM and substrate concentrations ranging from 2-200 nM, with analysis as above. The K_m^{app} and k_2 for the ribozymes is estimated for a Hanes plot with $R2 > 0.90$. Catalytic efficiency is calculated as k_2/ K_m^{app}. In vitro cleavage data for several representative ribozymes targeting specific sites in the CDK4, CDK2, CDC2, and cyclin B1 genes is shown in table 10.

Table 10

Summary of kinetics data for additional hairpin (HP) and hammerhead (HH) ribozyme candidates.

	HH k_2/K_m^{app}	HP k_2/K_m^{app}
CDK4		
cdk4-4 8.9	8.33	
cdk4-4,8g6		7.3
cdk4-1 7.9	6.61	
CDC2		
cdc2-6/ 7,8 g7h	14.4 pig, 31.9 hu	
cdc2-6,8g7h		6.25
CDK2		
CDK2-4 /7,9	27.37	
CDK2-4,7		10.76
CYCB1		
CycB 8.8	9.7	

EXAMPLE 6

IN VIVO USE OF RIBOZYMES

A. Experimental Protocol

All animals are treated according to the guidelines of the American Physiological Society. Briefly, a #2 Fr fogarty catheter is used to induce vascular injury in male Sprague-Dawley rats (400 to 500 g in weight). The rats are anesthetized and a cannula is introduced into the left common carotid artery via the external carotid artery. The common carotid artery is then injured by pulling the inflated fogarty catheter through it 3 times. A total of 100 animals are studied and divided into 6 different groups, as set forth below in Table 11:

Table 11

Group 1	(n=20)	balloon injury alone.
Group 2	(n=15)	balloon injury followed by infusion of saline through an isolated segment.
Group 3	(n=15)	balloon injury followed by local administration of CDC2 kinase ribozyme.
Group 4	(n=12)	balloon injury followed by local delivery of ribozyme to PCNA.
Group 5	(n=25)	balloon injury followed by administration of scrambled sequences of nucleotides resembling CDC2 kinase and PCNA ribozymes.
Group 6	(n+12)	balloon injury followed by local administration of a combination of CDC2 kinase and PCNA ribozymes.

After vessel injury of the common carotid artery, the injured segment is transiently isolated by temporary ligatures. Liposomes are used to encapsulate the ribozymes for delivery at the site of injury. Preferred liposomes include DOTAP:cholesterol (USSN 60/024,386, "Novel DNA:Liposome Complexes for Increased Systemic Delivery and Gene Expression", Smyth-Templeton, N et. al.),

Lipofectin (US 4,897,355, "Eppstein et. al.), and LT1 (Mirus Corp., Madison WI). Briefly, two hundred microliters of a combination of liposome and synthetic ribozyme (40 μg) are incubated in the isolated segment for 15 minutes. After the 15 minute incubation, the ligatures are removed. The external carotid artery is ligated and blood flow is restored in the common carotid and the internal carotid artery. The skin wound is then repaired and the animals are transferred to their cages. The animals are then euthanized at 2 weeks and artery is harvested. It s perfusion fixed in formalin and sent for histopathology.

The histopathology sections are then subsequently analyzed by quantitative histology. Using computer facilitated planimetry, the lumen area, area of the intima and area of the media are measured and intimal area to medial area ration is calculated. All values are expressed as mean ± standard deviation and mean ± standard errors of mean. A statistical comparison for each of these parameters is performed between all the groups.

Results of the quantitative histology are shown in Figures 6 and 7 and summarized in Table 12. Briefly, both the cross-sectional area of the intima and the ratio of the intimal area to medial area were significantly reduced in the ribozyme treated arteries compared with those treated with scrambled-sequence polynucleotides or with normal saline. The intimal hyperplasia was inhibited by the CDC-2 kinase ribozyme, the PCNA ribozyme and their combination. The combination did not seem to have any additive effect.

Table 12

	NO.		INT	I/M
B1	14	MEAN	13.50	0.83
		STDEV	4.47	0.34
B1+NS	8	MEAN	17.74	1.09

	NO.		INT	I/M
		STDEV	6.52	0.42
B1+RZ1	18	MEAN	8.37	0.46
		STDEV	5.04	0.24
B1+SCR	19	MEAN	13.24	0.92
		STDEV	4.43	0.26
B1+RZ2	10	MEAN	7.21	0.43
		STDEV	3.87	0.24
B1+RZoom	10	MEAN	6.218783	0.41197
		STDEV	1.875044	0.141841

B. Additional Assays

1. Tissue Culture Protocols

Smooth muscle cells (SMC) are isolated from rat aorta and maintained in DMEM medium and 10% FBS. MTT assay: This is a quantitative colorimetric assay for cell proliferation and survival. Rat SMC's (passage 4-8) are seeded into 96 well plate (1500 cells/well) one day before treatment. Cells are then treated with 2 mM of CDC-2 kinase/PCNA ribozyme and 4 mM lipofectin for 1 hour. A second dose of ribozyme (4 mM) is added on day 2. On day 3, 10 mL of MTT is added into each well for 4 hours. The dye in the cells is extracted in DMSO after washing off any supernatant dye from the well. The OD is measured with microplate reader at 590 mM.

The MTT assay using PCNA ribozyme demonstrates significant inhibition of cell proliferation in cell culture as measured by uptake of MTT in comparison to scrambled sequence treated cells and control cells.

2. Quantification of mRNA

SMC's (4-8 passage) are seeded into culture dish one day prior to treatment. RNA is extracted from the cells after treatment with ribozyme, scrambled sequence polynucleotide, 10% FBS or serum free medium for 2 or 6 hours. RT-PCR is then performed utilizing RNA-PCR kit from Perkin Elmer. An appropriated primer sequence for CDC-2 kinase or PCNA is used for analysis. A beta-actin primer is used to ensure that the amount of RNA loaded in each well is approximately equal.

RT-PCR studies using CDC-2 kinase ribozyme show reduction in the CDC-2 kinase mRNA at 2 hours and further reduction at 6 hours in comparison to controls. To ensure that equivalent amount of RNA is loaded in each well, RT-PCR is performed using a primer for beta-actin which shows similar levels of beta-actin mRNA in each group.

3. Protein Expression

Three types of protein assays may also be accomplished, including a) Western blotting; b) Biosynthetic labeling with 35S labeled methionine followed by immunoprecipitation of radiolabelled protein as a measure of newly synthesized target protein; and c) Histone H1 kinase assay for CDC-2 kinase. The Histone H1 kinase assay is a functional assay for CDC-2 kinase and measures the amount of p32 labeled phosphate transferred from ATP to Histone H1.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. A hairpin or hammerhead ribozyme which cleaves RNA encoding with a cyclin or cell-cycle dependent kinase, with the proviso that said cell-cycle dependent kinase is not CDK1, PCNA or Cyclin B1.

2. The ribozyme according to claim 1 wherein said ribozyme cleaves either CDK4 or CDK2.

3. The ribozyme according to claim 1 wherein said ribozyme cleaves Cyclin D.

4. The ribozyme according to claim 1 wherein said ribozyme is composed of ribonucleic acids.

5. The ribozyme according to claim 4 wherein one or more of said ribonucleic acids are 2'-O-methyl ribonucleic acids.

6. The ribozyme according to claim 1 wherein said ribozyme is composed of a mixture of deoxyribonucleic acids and ribonucleic acids.

7. The ribozyme according to claim 1 wherein said ribozyme is composed of nucleic acids having phosphothioate linkages.

8. A nucleic acid molecule encoding the ribozyme of claim 1.

9. The nucleic acid molecule of claim 8, wherein the nucleic acid is DNA or cDNA.

10. The nucleic acid molecule of claim 8, under the control of a promoter to transcribe the nucleic acid.

11 A host cell comprising the ribozyme of claim 1.

12. A vector comprising the nucleic acid of claim 8.

13. The vector of claim 12, wherein the vector is a plasmid, a virus, retrotransposon or a cosmid.

14. The vector of claim 13, wherein said virus is selected from the group consisting of retroviruses, adenoviruses, and adeno-associated viruses.

15. The vector according to claim 13 wherein said vector is generated from two or more different viruses.

16. A host cell comprising the vector of claim 12.

17. The host cell according to claim 16 wherein said host cell is stably transformed with said vector.

18. The host cell according to claim 16 wherein the host cell is a human cell.

19. A method for producing a ribozyme, comprising providing DNA encoding the ribozyme under the transcriptional control of a promoter, and transcribing the DNA to produce the ribozyme.

20. The method of claim 19, wherein the ribozyme is produced *in vitro*.

21. The method of claim 19, further comprising purifying the ribozyme produced.

22. The method of claim 19, wherein the ribozyme is produced *in vivo*.

23. The method according to claim 19 wherein said DNA encoding a ribozyme is a recombinant viral vector which directs the transcription of said ribozyme.

24. The method according to claim 19 wherein said DNA encoding a ribozyme is a plasmid vector which directs the transcription of said ribozyme.

25. A method of inhibiting restenosis, comprising introducing into a cell an effective amount of the ribozyme of claim 1.

26. A method of inhibiting restenosis, which comprises introducing into the cell an effective amount of the ribozyme according to claim 1.

27. The method of claim 20 or 25 wherein the cell is a human cell.

28. A method of preventing restenosis, which comprises introducing into the cell an effective amount of the DNA of claim 8 under conditions favoring transcription of the DNA to produce the ribozyme.

29. The method of claim 28, wherein the cell is a human cell.

30. The method according to claims 26 or 28 wherein the ribozyme is delivered to the cell exoluminally, or, transluminally.

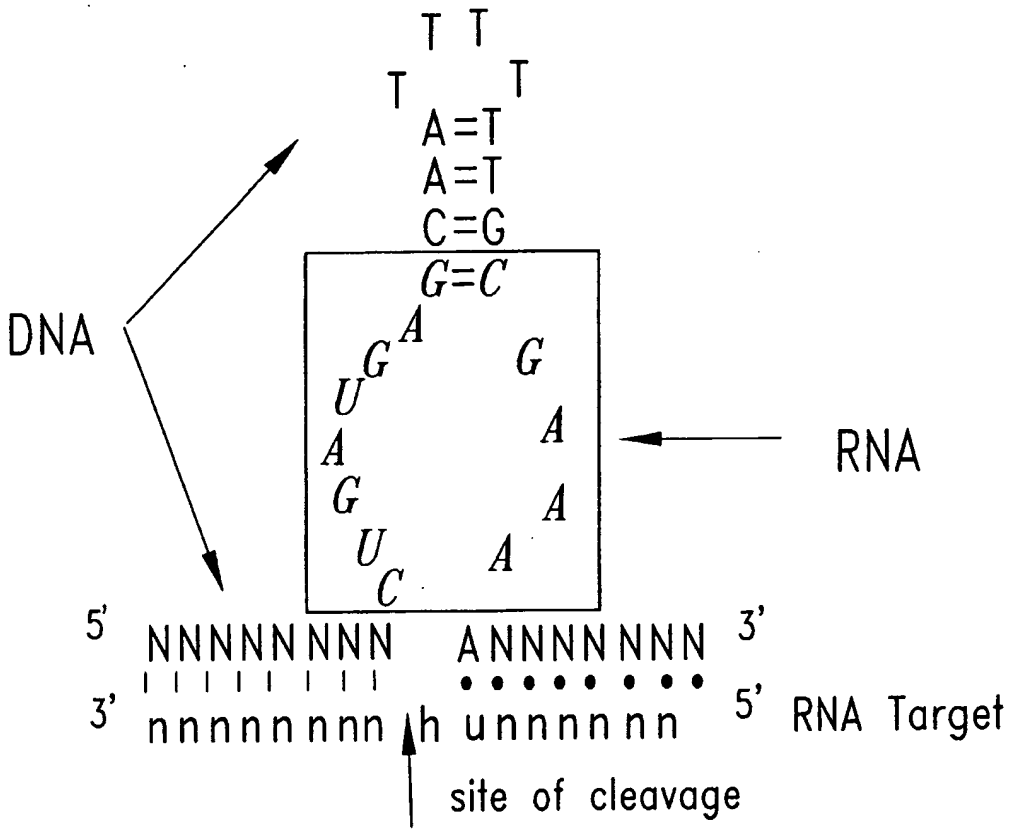
31. The method according to claims 26 or 28 wherein the ribozyme is delivered to the cell by catheter, stent, by a biodegradable polymer or sphere or in a pleuronic gel.

32. A pharmaceutical composition, comprising the ribozyme according to claim 1 and a pharmaceutically acceptable carrier or diluent.

33. The pharmaceutical composition according to claim 32 wherein said carrier is a lipid.

34. The pharmaceutical composition according to claim 33 wherein said lipid is DOTAP:cholesterol.

Fig. 1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A=T
A=T
C=G
G=C
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RNA
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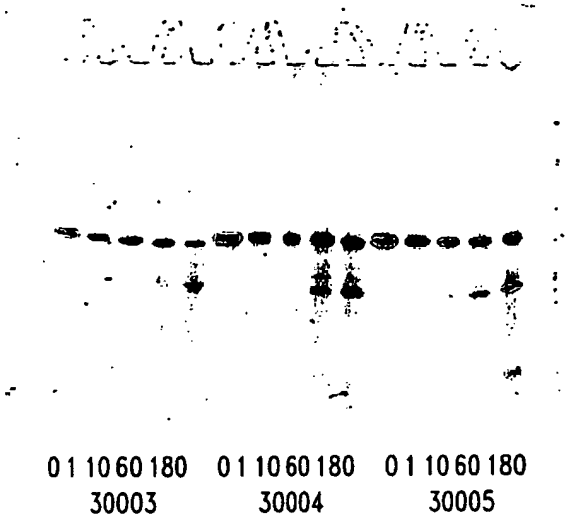
3' nnnnnnnnn h unnnnnnn 5' RNA Target
site of cleavage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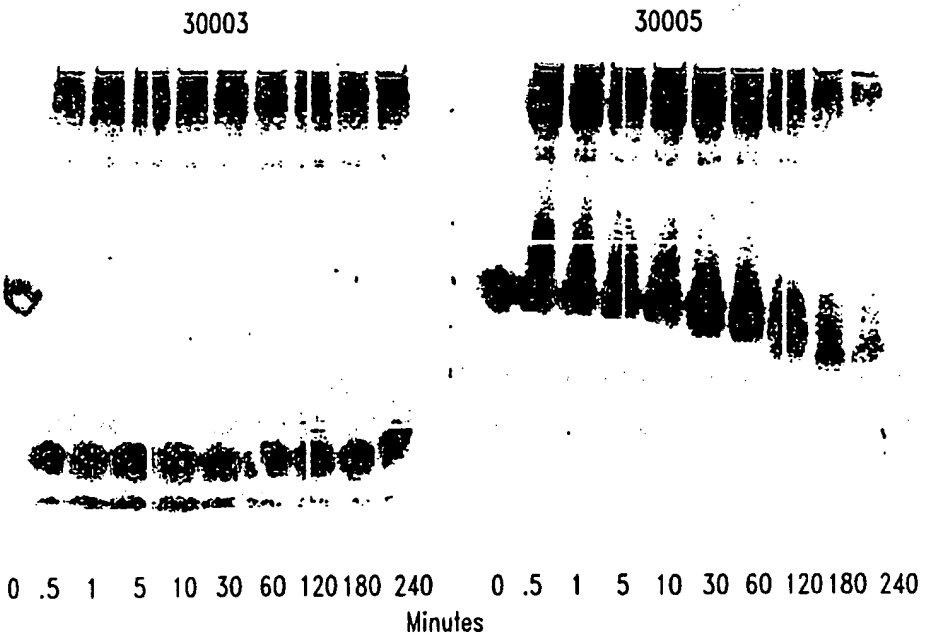
30003 30004 30005

Fig. 2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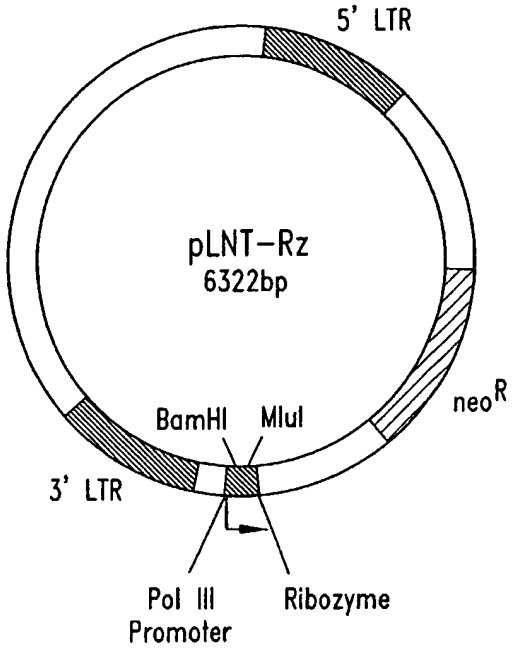
Minutes

Fig. 3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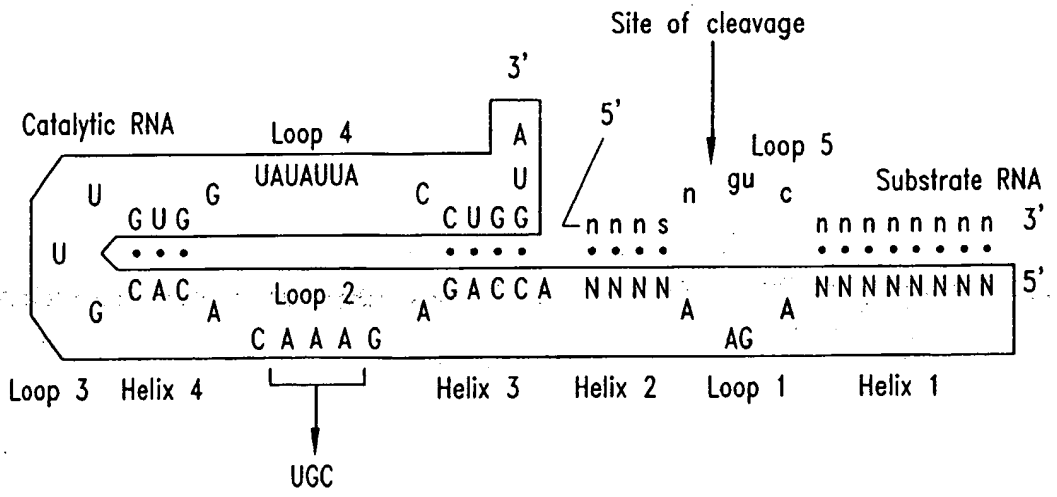
neo^R
BamHI
MluI
3' LTR
Pol III
Promoter
Ribozyme

Fig. 4


Site of cleavage
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5'
Catalytic RNA
Loop 4
UAUAUUA
Loop 5
Substrate RNA
Loop 2
Loop 3
Helix 4
Helix 3
Helix 2
Loop 1
Helix 1
UGC

Fig. 5

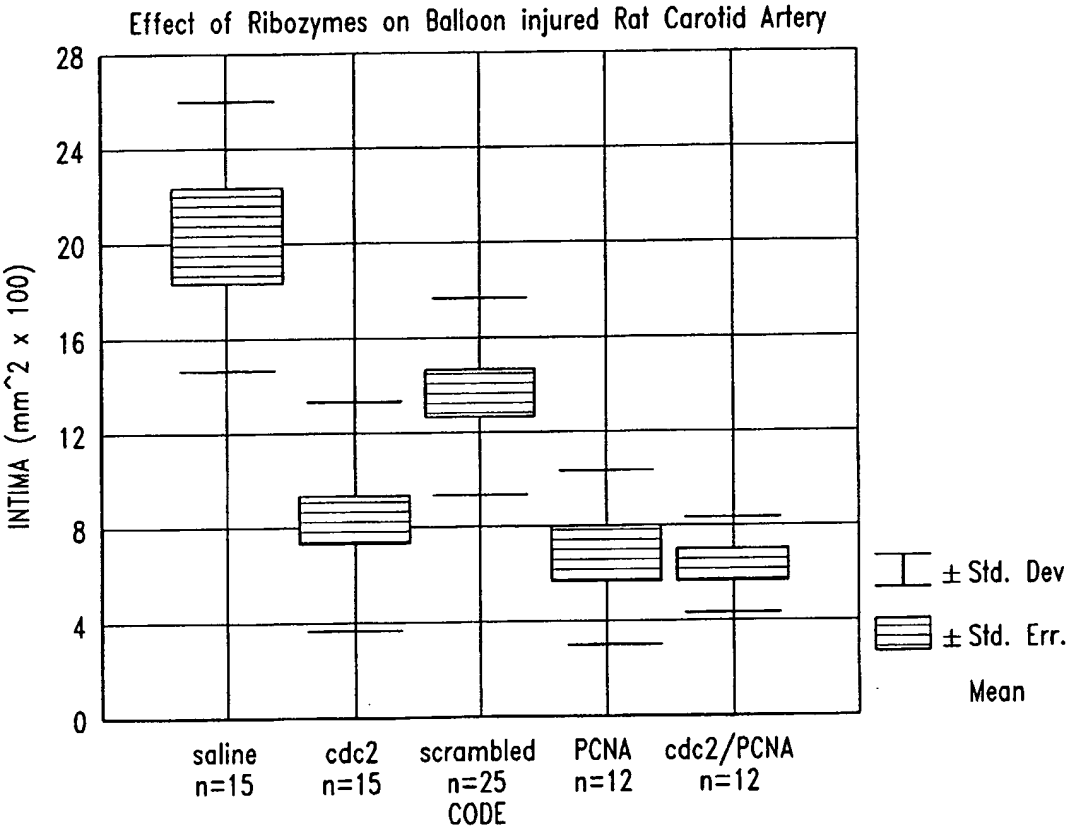
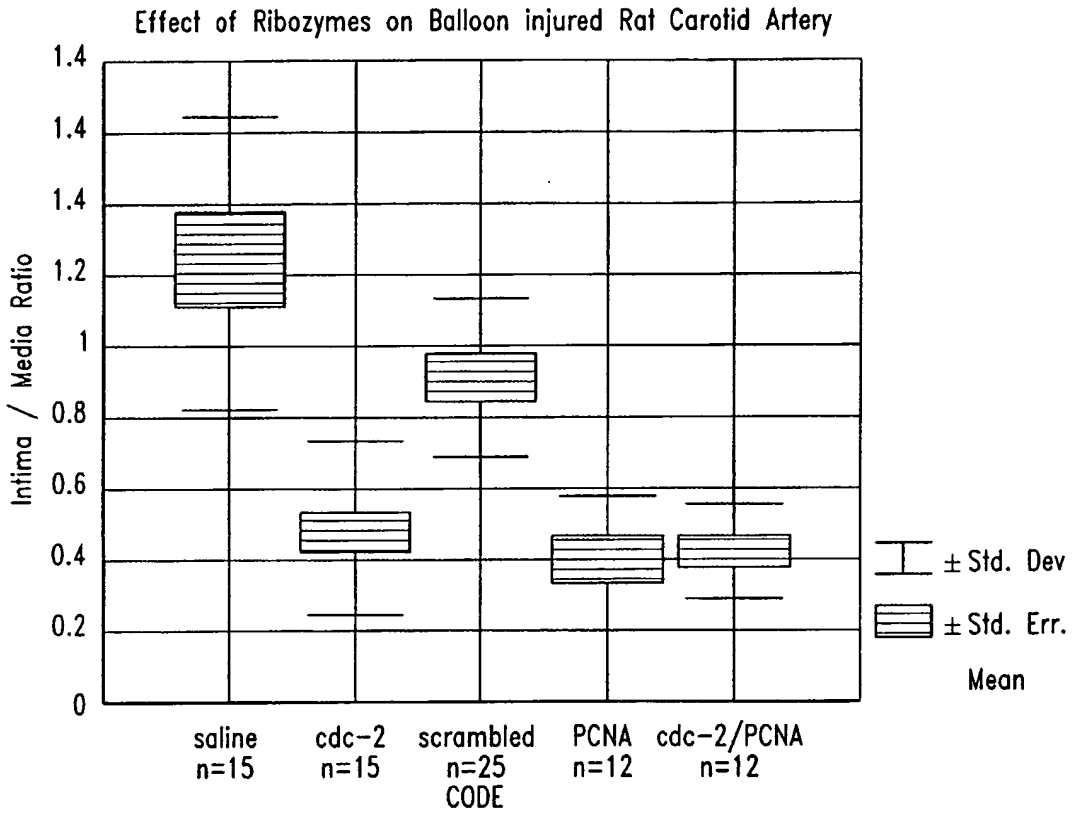

Effect of Ribozymes on Balloon injured Rat Carotid Artery
INTIMA (mm^2 x 100)
28
24
20
16
12
8
4
0
saline n=15
cdc2 n=15
scrambled n=25
PCNA n=12
cdc2/PCNA n=12
CODE
± Std. Dev
± Std. Err.
Mean

Fig. 6


Effect of Ribozymes on Balloon injured Rat Carotid Artery
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1.4
1.4
1.2
1
0.8
0.6
0.4
0.2
0
saline n=15
cdc-2 n=15
scrambled n=25 CODE
PCNA n=12
cdc-2/PCNA n=12
± Std. Dev
± Std. Err.
Mean

Fig. 7

SEQEUNCE LISTING

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acacactctc tgtgaaagt SEQ ID NO: 10
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tttactgttt gtgagagga SEQ ID NO: 17
gagtgtggta ctccactca SEQ ID NO: 18
tgtggtactc cactcagaa SEQ ID NO: 19
tactccactc agaaagagc SEQ ID NO: 20
agagcaactt cagggtaca SEQ ID NO: 21
gagcaacttc agggtacaa SEQ ID NO: 22
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tacaactctt tgaagatcc SEQ ID NO: 25
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INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/US 99/28772

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C12N9/00 C12N15/86 A61K48/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 10334 A (IMMUSOL INC) 20 March 1997 (1997-03-20) the whole document ---	1,4-32
X	GRASSI G ET AL: "Growth inhibition of smooth muscle cells from human coronary plaque tissues by hammerhead ribozymes" PATHOLOGY RESEARCH AND PRACTICE, vol. 194, 1998, page 267 XP000910797 abstract 214 --- -/--	1,4,8-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2000	31.08.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	CUPIDO, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern d Application No
PCT/US 99/28772

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEV V ET AL: "RIBOZYMES TO CELL DIVISION CYCLE (CDC-2) KINASE AND PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) PREVENT INTIMAL HYPERPLASIA IN RAT CAROTID ARTERY" CIRCULATION,US,AMERICAN HEART ASSOCIATION, DALLAS, TX, vol. 92, no. 8, 15 October 1995 (1995-10-15), page S34 XP000616561 ISSN: 0009-7322 abstract 3040 ---	1,4, 8-26,28
X	WO 94 26888 A (LELAND STANFORD JUNIOR UNIVERSITY) 24 November 1994 (1994-11-24) page 23, line 12 - line 13; claims 1,2 -----	2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In tional application No.
PCT/US 99/28772

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 25-31 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the ribozyme.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,2, 4-34 (all partly)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1,2, 4-34 (all partly)

A ribozyme which cleaves RNA encoding a cyclin or cell-cycle dependent kinase, wherein said ribozyme cleaves CDK4, nucleic acid encoding the ribozyme, host cells and vectors containing said nucleic acid or ribozyme, methods to produce said ribozyme, methods of inhibiting restenosis involving the use of said ribozyme and corresponding pharmaceutical compositions.

2. Claims: 1,2, 4-34 (all partly)

A ribozyme which cleaves RNA encoding a cyclin or cell-cycle dependent kinase, wherein said ribozyme cleaves CDK2, nucleic acid encoding the ribozyme, host cells and vectors containing said nucleic acid or ribozyme, methods to produce said ribozyme, methods of inhibiting restenosis involving the use of said ribozyme and corresponding pharmaceutical compositions.

3. Claims: 1,3, 4-34 (all partly)

A ribozyme which cleaves RNA encoding a cyclin or cell-cycle dependent kinase, wherein said ribozyme cleaves Cyclin D, nucleic acid encoding the ribozyme, host cells and vectors containing said nucleic acid or ribozyme, methods to produce said ribozyme, methods of inhibiting restenosis involving the use of said ribozyme and corresponding pharmaceutical compositions.

INTERNATIONAL SEARCH REPORT

ition on patent family members

Interr al Application No
PCT/US 99/28772

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9710334 A	20-03-1997	US 5834440 A	10-11-1998
		EP 0850301 A	01-07-1998
		JP 11514855 T	21-12-1999
WO 9426888 A	24-11-1994	US 5821234 A	13-10-1998
		CA 2163234 A	24-11-1994
		EP 0701609 A	20-03-1996
		JP 9507381 T	29-07-1997
		US 5869462 A	09-02-1999